

Characterization of chilling-shock responses in four genotypes of *Miscanthus* reveals the superior tolerance of *M. × giganteus* compared with *M. sinensis* and *M. sacchariflorus*

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- **Background and Aims** The bioenergy grass *Miscanthus* is native to eastern Asia. As *Miscanthus* uses C₄ photosynthesis, the cooler temperatures experienced in much of northern Europe are expected to limit productivity. Identification of genetic diversity in chilling tolerance will enable breeders to generate more productive varieties for these cooler regions. Characterizing the temporal relationships between photosynthesis, carbohydrate and molecular expression of relevant genes is key to understanding genotypic differences in tolerance or sensitivity.
- **Methods** To characterize chilling responses in four *Miscanthus* genotypes, plants were exposed to a sudden reduction in temperature. The genotypes studied comprised of two *M. sinensis*, one *M. sacchariflorus* and one inter-species hybrid, *M. × giganteus*. Changes in photosynthesis (A_{sat}), carbohydrate composition and the expression of target transcripts were observed following chilling-shock. After 4 d the decline in leaf elongation rate (LER) in the different genotypes was measured.
- **Results** Following chilling-shock the greatest decline in A_{sat} was observed in *M. sacchariflorus* and one *M. sinensis* genotype. Carbohydrate concentrations increased in all genotypes following chilling but to a lesser extent in *M. sacchariflorus*. Two stress inducible genes were most highly expressed in the genotypes that experienced the greatest declines in A_{sat} and LER. *Miscanthus × giganteus* retained the highest A_{sat} and was unique in exhibiting no decline in LER following transfer to 12 °C.
- **Conclusions** *Miscanthus × giganteus* exhibits a superior tolerance to chilling shock than other genotypes of *Miscanthus*. The absence of sucrose accumulation in *M. sacchariflorus* during chilling-shock suggests an impairment in enzyme function. A candidate transcription factor, *MsCBF3*, is most highly expressed in the most sensitive genotypes and may be a suitable molecular marker for predicting chilling sensitivity.

Key words: Carbohydrates, chilling, diurnal, gene expression, genotypes, *Miscanthus × giganteus*, *M. sacchariflorus*, *M. sinensis*, photosynthesis, bioenergy.

INTRODUCTION

The search for a sustainable substitute for fossil fuels has stimulated research into bioenergy crops. The criteria for such crops include low-input requirements, rapid accumulation of biomass and high yield. Perenniality is also advantageous, both economically and environmentally, as financial costs are saved on annual re-planting and tilling and environmentally by the sequestration of carbon in the soil (Clifton-Brown *et al.*, 2007). As a C₄ perennial rhizomatous grass with a high annual yield and low-input requirements (Lewandowski *et al.*, 2000), *Miscanthus* fulfils these criteria. As a C₄ plant *Miscanthus* exhibits high radiation, water- and nitrogen-use efficiencies (Lewandowski *et al.*, 2000). The penalty of C₄ photosynthesis, however, is a higher thermal requirement to initiate growth in the spring which imposes a limitation on growing range (Long, 1983; Naidu *et al.*, 2003). Currently all commercial

plantations of *Miscanthus*, and indeed a large amount of the research, have focussed on a single genotype *M. × giganteus*. This genotype is a sterile triploid progeny of a cross between *M. sinensis* and *M. sacchariflorus*; it is high yielding, reportedly producing over 20 t dry matter ha⁻¹ year⁻¹ in Europe (Lewandowski *et al.*, 2000) and over 30 t dry matter ha⁻¹ year⁻¹ in the mid-western USA (Dohleman and Long, 2009). An unusual characteristic of *M. × giganteus* is its ability to maintain high rates of light-saturated photosynthesis under chilling conditions inhibitory to other C₄ crops such as *Zea mays* (maize; Naidu *et al.*, 2003). However, it has shown poor over-winter survival rates in northern locations (Clifton-Brown and Lewandowski, 2000) and, owing to its sterility, *M. × giganteus* can only be vegetatively propagated from rhizome pieces, resulting in high establishment costs. A major plant breeding target is to develop a seed-based crop that can equal or outperform *M. × giganteus*.

The geographical range for *Miscanthus* spans from the subtropics to the sub-arctic (Numata, 1974; Clifton-Brown and Lewandowski, 2000). Correspondingly, variation in cold tolerance has been observed in different genotypes (Clifton-Brown and Jones, 1997; Clifton-Brown and Lewandowski, 2000; Farrell *et al.*, 2006; Yan *et al.*, 2012). Cold tolerance may refer to the ability of a rhizome to survive winter conditions (Clifton-Brown and Lewandowski, 2000; Yan *et al.*, 2012), late spring frosts (Farrell *et al.*, 2006), seedling survival (Yan *et al.*, 2012), the continuation of leaf expansion in progressively colder temperatures (Clifton-Brown and Jones, 1997) or the ability to retain high rates of photosynthesis under cool conditions (Wang *et al.*, 2008a). In spring when the canopy is still developing the daily temperature fluctuations in northern European locations are typically near freezing at night and up to 30 °C on a clear day. Temperatures may vary greatly between days but also within the daylight period of individual days. For example, in the coastal region of West Wales, temperatures in the daylight period on 22 May 2010 were recorded to decrease 14 °C between the warmest time of day (28 °C, at approximately noon) and sunset (approximately 2100 h; Supplementary Data Table S1). Sub-optimal temperatures during the light period depress photosynthetic performance of maize, and complete recovery may take several days even after a return to favourable temperatures (Andrews *et al.*, 1995). Whether all *Miscanthus* genotypes are equally susceptible to the same limitations remains to be determined. For the production of *Miscanthus* in the cooler climates of northern Europe and parts of North America all of these aspects will have to be considered to generate high yielding varieties. *Miscanthus sinensis* has been reported to have greater tolerance to cold, in terms of overwinter survival, compared with *M. × giganteus* (Clifton-Brown and Lewandowski, 2000). However, *M. × giganteus* has been shown to retain high levels of photosynthesis compared with other C₄ grasses under cool conditions (Naidu *et al.*, 2003; Wang *et al.*, 2008a; Dohleman and Long, 2009; Dohleman *et al.*, 2009), although how this compares with other *Miscanthus* genotypes is yet to be reported.

Miscanthus is a bioenergy crop harvested for the structural carbohydrates in the cell wall. Carbohydrates assimilated through photosynthesis may be directly respired, converted into structural forms (cellulose and hemi-cellulose), maintained in soluble form or converted into the transient storage form, starch. Carbohydrate partitioning refers to the division of carbohydrates between these pools. To date there have been no studies that address the non-structural carbohydrate status of the above-ground material in either optimal or short-term chilling conditions in *Miscanthus*. The changes in carbohydrate status that occur in response to chilling have been documented in a number of other species including maize (Hodges *et al.*, 1997), *Saccharum* sp. (sugarcane) (Du and Nose, 2002), *Arabidopsis thaliana* (arabidopsis) (Stitt and Hurry, 2002) and *Helianthus annuus* (sunflower) (Paul *et al.*, 1991). The changes in the non-structural carbohydrate pools can be indicative of chilling tolerance or susceptibility. For example, chilling tolerant varieties of sugarcane accumulated soluble sugar in response to chilling, whereas a sensitive variety did not (Du and Nose, 2002). In maize the opposite response was observed (Hodges *et al.*, 1997). In a chilling-

tolerant plant such as arabidopsis, chilling-shock leads to a shift in carbohydrate partitioning away from starch and into soluble sugars (Kaplan *et al.*, 2004). The breakdown of starch during chilling leads to an increase in soluble sugars, such as maltose, which have protective properties during temperature stress (Kaplan *et al.*, 2004). Whether this adaptive response also occurs in *Miscanthus* remains to be determined.

Upon exposure to chilling temperatures, large transcriptional changes have been documented to take place in a number of species including *Vitis vinifera* (grapevine) (Tattersall *et al.*, 2007), arabidopsis (Provart *et al.*, 2003; Hannah *et al.*, 2006; Kaplan *et al.*, 2007), maize (Trzcinska-Danielewicz *et al.*, 2009) and sugarcane (Nogueira *et al.*, 2003). Transcriptional changes that take place during chilling in *Miscanthus* are yet to be studied. The identification of DNA-based markers of chilling tolerance (or susceptibility) would facilitate the screening of seedlings or potential parents to accelerate the breeding of new varieties better adapted to more northerly locations.

In temperate climates such as northern Europe and the mid-western USA, short-term temperature deviations from favourable temperatures to chilling are problematic for thermophilic C₄ crops (Andrews *et al.*, 1995; Allen and Ort, 2001). Studies in other species, including maize, arabidopsis, *Triticum aestivum* (wheat) and *Hordeum vulgare* (barley), have successfully used chilling-shock experiments to identify cold-inducible transcripts and transcription factors (Cattivelli and Bartels, 1989; Liu *et al.*, 1998; Medina *et al.*, 1999; Shen *et al.*, 2003; Nguyen *et al.*, 2009), metabolic responses to chilling (Kaplan *et al.*, 2004) and cold-signalling molecules (Knight *et al.*, 1996). Therefore, to investigate chilling-shock responses, we performed an extreme version of the cooling experienced on 22 May 2010 in West Wales (Table S1) by exposing plants to a rapid reduction in temperatures from 28 °C to 12 °C.

We conducted a twin-time course in which plants were measured over 12 h at optimal growing conditions and then over the same time period after an abrupt shift to chilling conditions. On 22 May 2010, the daily maximum temperature reached 28 °C at the field site in West Wales (Table S1). All genotypes used in this study are capable of flowering under a constant temperature of 28 °C (C. Hayes, pers. comm., Aberystwyth University, UK), probably because this is a temperature regime representative of their native environment in Japan. The *M. sacchariflorus* genotype used in this study has never been reported to flower under field conditions in West Wales. We therefore concluded that 28 °C was representative of both a northern European high temperature and also an optimal (non-restrictive) temperature for growth of all genotypes tested. To select a chilling regime we used the results of previous studies as a basis: Allen and Ort (2001) describe chilling conditions in C₄ crops to be 0–12 °C and in a study into chilling tolerance in *M. × giganteus*, Naidu *et al.* (2003) used a chilling treatment of 14/11 °C. We therefore chose a compromise between these two reports of 12 °C to impose chilling. We characterized the changes in photosynthesis, soluble sugars and starch following chilling-shock and then, to investigate the molecular response to chilling, we conducted qRT-PCR on target transcripts. Amongst the plethora of genes documented to be affected by chilling in other species we

focussed on the expression of genes involved in carbohydrate metabolism, cold signal transduction and stress responses. The genome of *Miscanthus* has not yet been sequenced so we preferentially selected genes for study that had been annotated in maize or sorghum genome databases. Finally, we measured changes in leaf-extension rates in plants subjected to chilling treatments compared with control plants maintained at 28 °C.

The aim of our experiments was 3-fold: (1) to characterize photosynthesis and carbohydrate dynamics throughout the light period in different genotypes of *Miscanthus*; (2) to measure changes in photosynthesis, carbohydrates and gene expression during chilling-shock; and (3) to identify suitable measurements or markers that could, in future, be used to screen prospective parent plants or crosses for chilling-shock tolerance/sensitivity.

MATERIALS AND METHODS

Plant material

All genotypes used in this study are of Japanese origin. A summary of geographical origin, ploidy and phenotypic attributes for each genotype are provided in Table 1. *Miscanthus sinensis* (EMI-11) is a diploid clone selected in 1988 from temperate Japan (Honshu Island) by Danish plant collector Dr Poul Erik Brander. It was part of the European *Miscanthus* Improvement (EMI) programme (1997–2000) and is the female parent of the Mx2 mapping family (Ma *et al.*, 2012). *Miscanthus sinensis* (Goliath) is an intraspecific hybrid of *M. sinensis*. It is a triploid, originally selected as a vigorous seedling from a cross (parents unknown) by Ernst Pagels and marketed as a ‘large-type’ horticultural variety since the 1970s. *Miscanthus sacchariflorus* (Sac-5) is a tetraploid that was part of a seed population collected from central Japan by TINPLANT in 1992. It was also part of the EMI programme. *Miscanthus* × *giganteus* (Gig-311) is a naturally occurring hybrid of diploid *M. sinensis* and a tetraploid *M. sacchariflorus*. It was supplied to IBERS from Bical (Taunton) in 2005.

Growth conditions and treatments

Plants were propagated from rhizome pieces cut from field-grown mature plants in December 2010. Rhizome pieces were grown in a polytunnel under natural lighting in 1-L pots in moist compost placed on capillary matting to ensure constant water availability. When emerged stems had reached four to seven mature (ligule) leaves on the tallest stem they were transferred to a growth room (Sanyo Fitotron) set at a constant (day

and night) temperature of 28 °C on a 12 h light : 12 h dark diurnal cycle with a light intensity of 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density (PPFD) for 10 d. Relative humidity was set at 84 % at 28 °C and 57 % at 12 °C to maintain a vapour pressure deficit of 0.6 kPa in both rooms. Plants were randomly arranged in trays containing six plants. Each tray contained a layer of capillary matting to ensure a constant availability of water. Plants were watered every 3 d during which time the plants were re-randomized within trays and the trays re-randomized within the growth room. After 10 d the time course experiment commenced. On day 1, plants were harvested at 0 h (lights on), 2 h, 4 h, 6 h, 10 h and 12 h (lights out). On day 2, 2 h after the lights came on half the plants were rapidly transferred to an identical controlled-environment room, with identical lighting settings but at a temperature of a constant 12 °C (day and night). Harvests at 12 °C were carried out at 4 h (2 h after transfer), 6 h (4 h after transfer), 10 h (8 h after transfer) and 12 h (10 h after transfer). A total of 33 plants per genotype were used in the experiments. Conditions in both rooms were monitored by in-built PC-based monitoring systems (SpecView Corp. and SpecView Ltd) and independent data loggers within the growth rooms monitored temperature and humidity (Rotronic International) and photosynthetically active radiation (Skye, Llandrindod Wells, UK).

Photosynthesis measurements

Leaf light-saturated CO₂ assimilation rates (A_{sat}) were measured *in vivo* using a portable open-path gas exchange system incorporating infrared CO₂ and water vapour analysers (Walz GFS-3000, Walz GmbH, Germany) incorporating a red–blue LED light source (Walz 3055-FL). Reference [CO₂] in the cuvette was set at 385 $\mu\text{mol mol}^{-1}$, the leaf temperature was maintained at the experimental temperature (12 °C or 28 °C), and vapour pressure deficit maintained between 1.1 and 2 kPa for all of the measurements (Long and Bernacchi, 2003). Three plants per genotype were randomly selected at the start of the experiment and measured at each time-point. Measurements were made on the youngest fully expanded leaf with a fully formed ligule and taken on the same area of the leaf, one-third of the leaf length from the leaf tip.

For each of the genotypes studied, a preliminary assessment of the response of photosynthesis to light was made on a small number of replicates to identify the PPFD required to reach A_{sat} . Plots of A vs. Q were measured starting at PPFD 2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and decreasing stepwise to complete darkness; curves consisted of a minimum of ten measurements of Q . Completed A vs. Q curves were fitted to a non-rectangular

TABLE 1. Description and phenotypes of the four genotypes used in this study

Genotype	Name	Ploidy	Height to highest ligule leaf (cm)	Stem no.	Geographical origin
<i>M. sinensis</i>	EMI-11	2n	13.6 ± 0.65 ^a	6.38 ± 1.27 ^a	Japan (temperate)
<i>M. × giganteus</i>	Gig-311	3n	50.3 ± 6.7 ^b	2.5 ± 0.33 ^b	Unknown
<i>M. sinensis</i>	Goliath	3n	29.3 ± 2.8 ^c	4.75 ± 0.62 ^a	Unknown
<i>M. sacchariflorus</i>	Sac-5	4n	55.7 ± 7.6 ^b	2.5 ± 0.33 ^b	Japan (central)

Measurements of height and stem number are the average of eight plants per genotype from rhizome-propagated plants (± s.e.). Different letters show significant differences between genotypes (Student’s two-tailed *t*-test, $P < 0.05$).

empirical function to estimate A_{sat} , which was consistently attained at a PPFD of $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Bernacchi *et al.*, 2003).

For the measurement of A_{sat} the leaf was placed in the gas analyser leaf chamber with the PPFD set at ambient irradiance; all other chamber parameters were as described above. The PPFD was then increased stepwise to $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ then to $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$. Each leaf was first allowed to achieve steady-state CO_2 and water vapour exchange at each increase of Q (Bernacchi *et al.* 2003). A_{sat} was measured at a PPFD of $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ once the steady state had been achieved (Wang *et al.*, 2012).

Collection of tissue for RNA and carbohydrate analysis

For each genotype three biological replicates (separate plants) were sampled per time-point, per treatment. The same leaf was used for both RNA and carbohydrate analysis but a separate set of plants were used for measuring photosynthesis.

Samples for RNA extraction. At each time-point, two 2-cm sections from the middle of the highest ligule leaf, on either side of the central mid-rib (to facilitate grinding), were quickly excised with a razor, placed in a 2-mL micro-centrifuge tube and flash frozen in liquid nitrogen. Immediately afterwards the carbohydrate sample was harvested from the same leaf and flash frozen in liquid nitrogen. Four time-points were selected for transcriptional studies: 2 h, 4 h, 6 h and 10 h for plants grown at 28°C and 4 h (2 h after transfer), 6 h (4 h after transfer) and 10 h (8 h after transfer) at 12°C . Samples were stored at -80°C until processed.

Samples for carbohydrate analysis. An 8-cm section of leaf tissue on either side of the section harvested for RNA was cut out with a razor blade, wrapped in aluminium foil and flash frozen in liquid nitrogen. In addition to the time-points on which tissue was harvested for RNA, there were two other time-points, 0 h (lights on) and 12 h (lights off) when tissue was harvested for carbohydrate analyses. Carbohydrate samples were freeze-dried for 48 h (Freezemobile 6; Virtis Co Inc., Gardiner, NY, USA) then cryomilled to a fine powder (Spex SamplePrep 6870 Large Freezer Mill; www.spexsampleprep.com). Three biological replicates (individual plants) for each genotype were harvested at each time-point. A total of 30 plants per genotype were harvested for carbohydrate and/or RNA extraction.

Carbohydrate analysis

Soluble sugar extraction. Approximately 20 mg (actual weight recorded) of each cryo-milled carbohydrate sample was weighed into 2-mL screw-cap micro-centrifuge tubes. Sugars were extracted in 1 mL of 80% (v/v) ethanol heated in a water bath at 80°C for 20 min. Samples were then cooled and centrifuged and the supernatant extracted and added to a 15-mL tube. The process was repeated a further three times. A 0.5-mL aliquot of the supernatant containing the soluble sugars was added to a 2-mL micro-centrifuge tube and, along with the remaining pellet containing the insoluble fraction (including starch), dried down in a centrifugal evaporator (Jouan RC 1022, Saint Nazaire, France) until all the solvent

had evaporated. The dried-down residue from the soluble fraction was then re-suspended in 0.5 mL of distilled water. The dried pellet was stored at -20°C until analysed for starch.

Soluble sugar analysis. Soluble sugars were quantified enzymatically by the stepwise addition of hexokinase, phosphoglucose isomerase and invertase (Jones *et al.*, 1977). Samples were quantified photometrically by measuring the change in wavelength at 340 nm, 15 min after the addition of each enzyme. Sucrose, glucose and fructose were then quantified from a standard curve.

Starch quantification. Starch was quantified using a modified Megazyme protocol (Megazyme Total Starch Assay Procedure, AOAC method 996.11; Megazyme International, Ireland). Briefly, the dried pellet was resuspended in 0.4 mL of 0.2 M KOH, vortexed vigorously and heated to 90°C in a water bath for 15 min to facilitate gelatinization of the starch. A total of 1.28 mL of 0.15 M NaOAc (pH 3.8) was added to each tube and vortexed before the addition of 20 μL α -amylase and 20 μL amyloglucosidase (Megazyme). After incubation at 50°C for 30 min, a 0.02-mL aliquot was extracted and combined with 0.6 mL of GOPOD reagent (Megazyme). A total of 0.2 mL of this reaction was assayed photometrically on a 96-well microplate at 510 nm against a water blank. Starch was quantified from a standard curve. For both soluble sugar and starch quantification a series of standards were included on each plate assayed. Each sample and standard was tested in duplicate.

RNA extraction and quantitative real time PCR

RNA samples were ground in liquid N using a ball mill (Tissuelyser MM300, Retsch, www.retsch.com). RNA was extracted using RNeasy Plant mini kit (Qiagen) according to the manufacturer's instructions and quantified on a NanoDrop (ND1000; Thermo Scientific; www.nanodrop.com). Contaminating genomic DNA was removed by DNase treatment with Turbo DNase Free (Ambion, Austin, TX, USA). cDNA was synthesized from 0.5 μg of total RNA with iScript cDNA synthesis kit (Bio-Rad Laboratories Inc.). qPCR was performed on an Applied Biosystems, 7500 real time PCR system (Life Technologies; www.appliedbiosystems.com) with QuantiFast SYBR Green (Qiagen), according to the manufacturer's instructions. Cycle conditions were 95°C for 5 min, then 40 cycles of 95°C for 10 s and a combined annealing/extension phase of 60°C for 1 min, followed by a melt curve analysis. A dissociation curve for each reaction was included to check for non-specific primer binding. In the absence of a sequenced genome for *Miscanthus*, primers were designed from the *Sorghum bicolor* (sorghum) and *Zea mays* (maize) cDNA libraries available from the NCBI website (<http://www.ncbi.nlm.nih.gov/>; see Table S2). Annotated sequences in sorghum and maize are available for all genes tested from the NCBI database (<http://www.ncbi.nlm.nih.gov/>). The crossing point threshold was calculated using the Applied Biosystems, 7500 real time PCR system software. The crossing point threshold of two technical replicates was used to calculate expression relative to an internal control gene. A number of control genes were tested based upon published data. Czechowski *et al.* (2005) identified *Yellow Leaf Specific8* (*YLS8*) as a superior reference gene for abiotic stress treatments in arabidopsis. An orthologue of this gene was

identified in maize and sorghum and found to be the most stably expressed in *Miscanthus* in both warm (28 °C) and chilling (12 °C) conditions.

Leaf extension rate (LER)

The plants were grown, arranged and maintained as described above. This phase of the experiment was initiated 24 h after the on-set on the chilling time course. At the start of the experiment a stem with four to seven mature ligule leaves was identified from six plants per genotype. With permanent marker, the highest ligule and the point at which this met the unfurling leaf sheath was marked. Treatments were as follows: 28 °C for 4 d (control) and 12 °C for 24 h (overlapping the chilling time course) before transfer back to 28 °C for a further 3 d and 12 °C for 4 d. After 4 d the distance between the marked ligule and the mark on the unfurling leaf was determined with callipers. Plants were re-arranged within their respective trays and shifted within the growth room every 3 d.

Statistical analyses

ANOVA's and Tukey's tests were carried out using GenStat version 13, *t*-tests were performed in Excel (Microsoft Inc.) and $\alpha < 0.05$ was applied for all analyses. Analysis for carbohydrate data was carried out as follows. At 28 °C, a two-way ANOVA was used to compare the effect of genotype and time into the photoperiod on total soluble sugar; a corresponding Tukey's test identified significant differences between genotypes for each time-point. At 12 °C, two-way ANOVAs and corresponding Tukey's tests were used for each genotype to determine significant differences between warm and chilled treatments for each time-point. For the average increase and percentage increase in soluble sugars after transfer, two, one-way ANOVAs with corresponding Tukey's tests were used to identify significant differences. When single variables were compared for morphological characteristics and LER, Student's two-tailed *t*-tests were performed assuming unequal variances ($\alpha < 0.05$; Excel, Microsoft Inc.).

RESULTS

Phenotypes of selected genotypes

The four genotypes were selected because they represent two distinct species of *Miscanthus*; *M. sinensis* and *M. sacchariflorus* and an inter-specific hybrid *M. × giganteus* (Table 1). The genotypes also represent a range of ploidy. Three of the genotypes used in this study; Gig-311, Goliath and Sac-5 have successfully been used in previous studies to investigate freezing tolerance (Clifton-Brown and Lewandowski, 2000; Farrell et al., 2006; Zub et al., 2011). Although *M. × giganteus* (hereafter referred to as Gig-311) is a hybrid between an *M. sacchariflorus* (4n) and an *M. sinensis* (2n) the physical measurements of stem numbers and height place it as being more physically similar to *M. sacchariflorus* clone Sac-5 (hereafter referred to as Sac-5) than the diploid *M. sinensis* clone EMI-11 (hereafter referred to as EMI-11). There were no significant differences between Gig-311 and Sac-5 for maximum ligule height or stem number, whereas both of these

genotypes were taller, with fewer stems than either EMI-11 or the triploid *M. sinensis* clone Goliath (hereafter referred to as Goliath). Both EMI-11 and Goliath had a similar number of stems with an average of 61 % and 47 %, respectively, more than both Gig-311 and Sac-5.

Photosynthesis under optimal temperatures and after transfer to chilling conditions

Measurement of light-saturated photosynthesis (A_{sat}) revealed that at 28 °C Gig-311 and Sac-5 exhibited higher rates of A_{sat} throughout the day than either of the *M. sinensis* genotypes (Fig. 1A). EMI-11 exhibited the lowest rate of A_{sat} after 4 h, thereafter showing a steady rate of decline. All genotypes exhibited a rapid decline in A_{sat} within the first 2 h after transfer to 12 °C. Gig-311 and Sac-5 continued to decline in A_{sat} until 6 h, and Goliath until 10 h (Fig. 1A). Gig-311 maintained the highest absolute A_{sat} . At the end of the time course (12 h) the rank order of A_{sat} was almost the same as at 28 °C with the two *M. sinensis* genotypes having the lowest rate and Gig-311 and Sac-5 having the highest rate. As a percentage of A_{sat} at 28 °C, *M. × giganteus* maintained the highest percentage; Goliath and Sac-5 experienced the greatest decline (Fig. 1B). At the end of the time course, all genotypes had, relatively, declined to the same extent, to approx. 65–68 % of their corresponding rate at 28 °C.

Soluble carbohydrate contents at 28 °C

The most abundant soluble sugar was sucrose, followed by glucose and then fructose in all the genotypes (Fig. 2A–C). In EMI-11 and Goliath, sucrose contents increased from 6 h and 4 h, respectively, to the end of the light period, whereas in Sac-5 sucrose concentrations peaked at 6 h and then declined. The separation in accumulation trend between the *M. sinensis* genotypes and Sac-5 and Gig-311 was more obvious in their accumulation of glucose: EMI-11 and Goliath reached peak concentrations at 10 h and 12 h, respectively, whereas Gig-311 and Sac-5 reached peak concentration at 6 h. Fructose concentrations for EMI-11 and Goliath were low throughout the experiment, dropping below detectable concentrations at 0 h, 10 h and 12 h in EMI-11. Fructose concentrations were higher in Sac-5 and Gig-311, again, peaking at 6 h. The genotypes with the highest concentration of total soluble sugars consistently were Sac-5 and Gig-311, followed, in order, by Goliath and finally EMI-11 (Table 2). This pattern is consistent with their respective rates of photosynthesis throughout the day; the genotype with the highest rate (Sac-5) accumulated the most soluble sugars and vice versa.

Changes in soluble carbohydrate content following transfer to chilling conditions

After transfer to 12 °C an increase in sucrose, glucose and fructose concentrations was observed in all genotypes, but to different extents (Fig. 2A–C). The greatest increase in sucrose, compared with the 28 °C treatment, was an 85 % increase in EMI-11 at 6 h (Fig. 2A and Table 3). The smallest change in sucrose was observed in Sac-5 where concentrations remained comparable to the 28 °C treatment throughout the experiment. Sac-5,

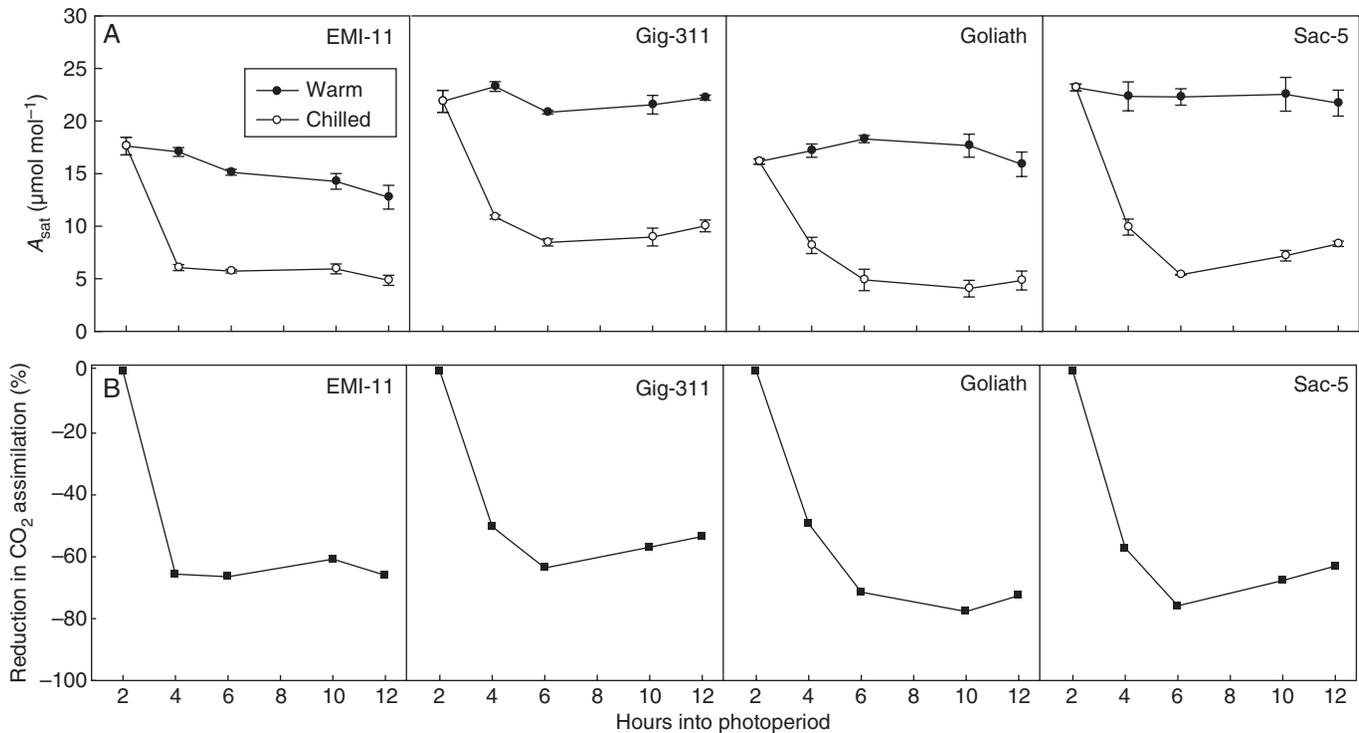


FIG. 1. (A) Light saturated photosynthesis (A_{sat}) at 28 °C (Warm) and after transfer to 12 °C (Chilled) in the four genotypes. (B) Percentage reduction in leaf CO_2 assimilation following transfer. Values are means \pm s.e., $n = 3$.

however, accumulated elevated concentrations of hexose in chilling conditions so the low concentrations of sucrose cannot be attributed to the impairment of photosynthesis (Fig. 2B). EMI-11 and Goliath, like Sac-5, accumulated large concentrations of glucose and fructose at 12 °C compared with 28 °C (B and C, respectively, in Fig. 2). Gig-311 accumulated the least concentration of hexose, with very little change in glucose content between the 12 °C and 28 °C treatments. Despite not accumulating hexoses to the same extent as the other genotypes, Gig-311, still exhibited the highest average concentration of soluble sugars (Table 3). In contrast to the absolute values, the percentage increase in soluble sugars revealed that EMI-11 and Goliath exhibited the greatest percentage increase in soluble sugars during chilling and Sac-5 showed the lowest. Gig-311 was intermediate between the two groups (Table 3).

Starch accumulation under optimal and chilling conditions

At 28 °C starch concentrations accumulated throughout the light period in all genotypes (Fig. 3). Although the total concentration of starch accumulated between the genotypes at the end of the day was similar, the pattern of accumulation was different. In the two *M. sinensis* genotypes starch accumulated fairly linearly throughout the day, whereas concentrations of starch remained low in Gig-311 and Sac-5 until 10 h when 71 % and 66 % of starch, respectively, was accumulated in the last 2 h of the light period (Fig. 3). In Gig-311 this pattern of accumulation mirrored that of sucrose which was also more abundant in the last 2 h of the light period (Figs 2 and 3).

After transfer to chilling conditions, starch continued to accumulate in all genotypes (Fig. 3). At 10 h, EMI-11, Gig-311 and Sac-5 all contained more starch at 12 °C than their warm-grown counterparts. In EMI-11 and Sac-5, a lower quantity of starch had accumulated at the end of the day in the chilled plants and concentrations had decreased in the last 2 h of the experiment.

Gene expression

To study molecular changes in response to chilling, transcripts homologous to those that had previously been identified as being involved in carbohydrate metabolism or stress responses in other species were identified in *Miscanthus* (Table 4). The pattern of expression for *MsPGMI* was similar for the two *M. sinensis* types showing a progressive increase in transcription from the point of transfer to 12 °C (Fig. 4A). Sac-5 and Gig-311 also had a very similar pattern of expression to each other but different from the two *M. sinensis* genotypes where expression peaked 2 h after transfer and then declined. The amplitude in expression of *MsPGMI* reflected the soluble sugar concentration; Sac-5 showed the slightest increase in expression of *MsPGMI* and the smallest increase in soluble sugars, whereas EMI-11 showed the greatest increase in soluble sugars and expression of *MsPGMI*. The expression patterns for *MsBAM3* (Fig. 4B) were similar to those of *MsPGMI*, which is interesting as they are most commonly regarded as being involved in opposite processes (breakdown and synthesis, respectively; for a review, see Zeeman *et al.*, 2007). The greatest increase in transcription of *MsBAM3*

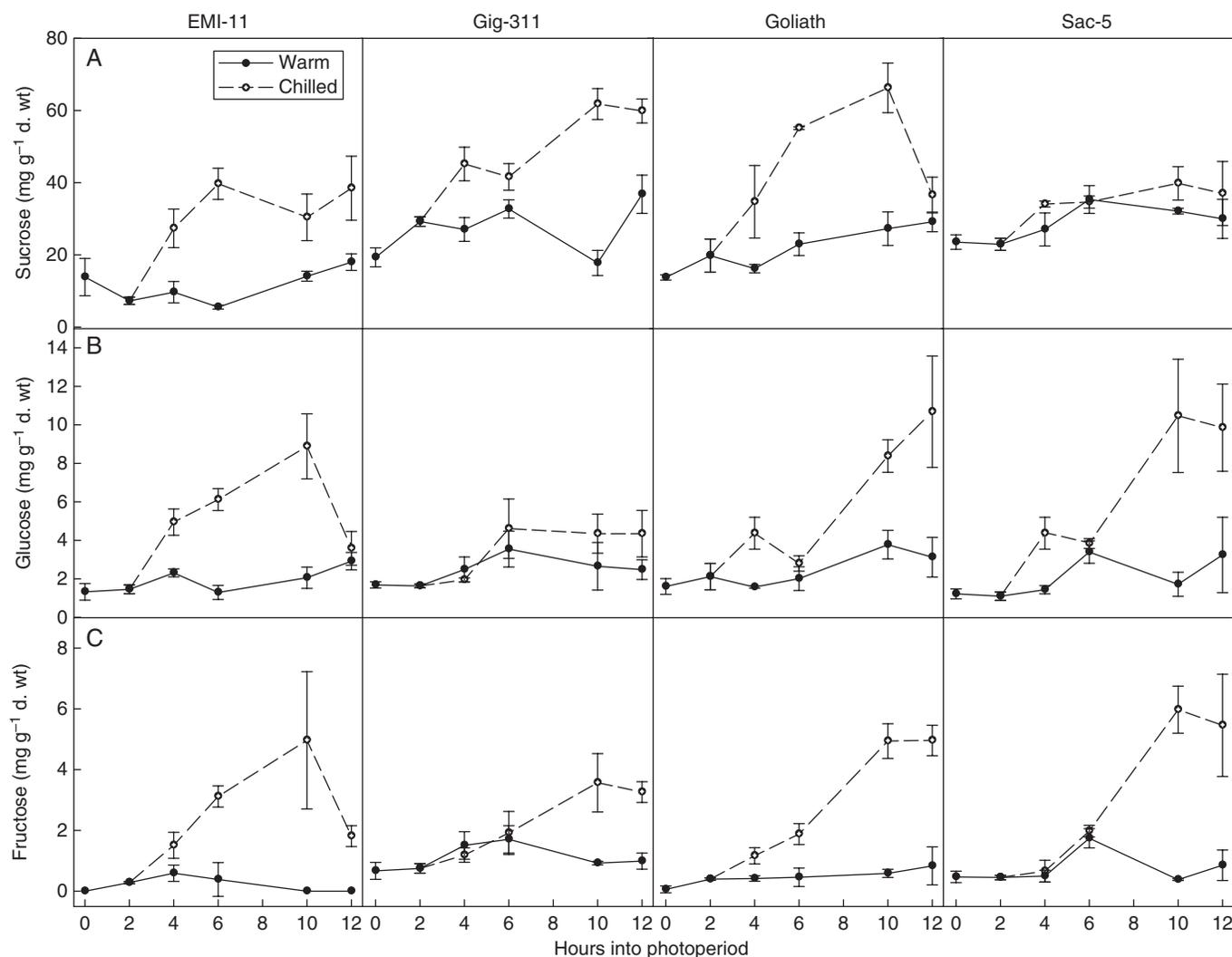


FIG. 2. Soluble sugar contents of the four genotypes at 28 °C (Warm) and after transfer to 12 °C (Chilled): sucrose (A), glucose (B) and fructose (C). Plants were transferred to 12 °C at 2 h. Values are means \pm s.e., $n = 3$.

TABLE 2. Average amount of total soluble sugars (glucose, sucrose and fructose; mg g⁻¹ d. wt) at 28 °C

Time into photoperiod (h)	EMI-11	Gig-311	Goliath	Sac-5
0	15.2 \pm 4.8 ^a	21.5 \pm 2.9 ^a	15.3 \pm 1.1 ^a	25.1 \pm 1.7 ^a
2	9.0 \pm 1.2 ^a	31.5 \pm 1.5 ^b	22.1 \pm 5.3 ^{ab}	24.3 \pm 1.8 ^b
4	12.5 \pm 3 ^a	30.9 \pm 4.4 ^b	18.0 \pm 1.1 ^{ab}	28.8 \pm 4.8 ^b
6	7.3 \pm 0.9 ^a	37.8 \pm 3.7 ^b	25.3 \pm 3.9 ^{ab}	40.3 \pm 4.5 ^b
10	16.1 \pm 1.9 ^a	21.3 \pm 4.6 ^{ab}	31.5 \pm 4.7 ^{ab}	34.0 \pm 1.4 ^b
12	21 \pm 2.7 ^a	40.1 \pm 5.0 ^a	32.9 \pm 3.8 ^a	33.9 \pm 7.1 ^a

Different letters show significant differences between genotypes (Tukey test, $P < 0.05$). $n = 3$, \pm s.e.

(8-fold) was observed 4 h after transfer to 12 °C in both EMI-11 and Sac-5. A lesser increase of 3- and 4-fold was observed in Goliath and Gig-311, respectively, at this same time-point (Fig. 4B). The fold change in expression pattern of *MsBAM3* and *MsPGMI* in the four genotypes mirrored the respective increases in sucrose accumulation at 12 °C (Figs 2A and 4A, B). A Pearson's Correlation co-efficient revealed average R^2 values of 0.4–0.9 for all genotypes, for both genes (A and B, respectively, in Fig. 5).

The C-repeat binding factors/dehydration responsive element binding (CBF/DREB) family of transcription factors have been proven to control the expression of a swathe of cold-inducible transcripts in arabidopsis (Yamaguchi-Shinozaki and Shinozaki, 1994; Gilmour *et al.*, 1998; Thomashow, 2010; Fowler *et al.*, 2005). The expression of this gene in *Miscanthus* showed the greatest fold increase in expression in response to cold of all the transcripts tested. Transcription was most highly expressed 2 h after transfer in all genotypes but this increase was transient

TABLE 3. Total soluble sugars (mg g⁻¹ d. wt) and percentage increase following transfer to 12 °C

Time into photoperiod (h)	EMI-11		Gig-311		Goliath		Sac-5	
	Total soluble sugars (mg g ⁻¹ d. wt)	% increase	Total soluble sugars (mg g ⁻¹ d. wt)	% increase	Total soluble sugars (mg g ⁻¹ d. wt)	% increase	Total soluble sugars (mg g ⁻¹ d. wt)	% increase
4	33.8 ± 6.4*	57	48.1 ± 4.7	33	40.1 ± 10.7	66	37.3 ± 0.7	18
6	48.9 ± 4.9*	85	47.9 ± 1.6	21	59.6 ± 0.6*	58	40.8 ± 1.9	0.1
10	44.3 ± 3.0*	63	69.5 ± 6.1*	69	79.4 ± 7.9*	60	56.1 ± 6.6	38
12	43.4 ± 9.1	45	67.3 ± 1.9*	40	50.5 ± 2.1	34	50.7 ± 12.4	19
Average	42.6 ± 3.2* ^b	62.5 ^b	58.2 ± 5.9* ^{ab}	40.8 ^{ab}	57.4 ± 8.4* ^b	54.5 ^b	46.1 ± 4.4* ^a	18.8 ^a

Mean total mg g⁻¹ d. wt glucose, fructose and sucrose following transfer to 12 °C and percentage increase from total amount of soluble sugar at 28 °C grown plants at the same time-point. $n = 3$, ± s.e.

* Significant difference from 28 °C. Different letters show significant differences between genotypes for the overall average and percentage increase (Tukey test, $P < 0.05$).

and, by the end of the experiment, the change was <6-fold for all genotypes (Fig. 4C). Goliath expressed *MsCBF3* most highly with a 56-fold change, followed by Sac-5, Gig-311 and EMI-11 which only increased 4-fold at the peak of expression.

RD19a is under strong circadian regulation (Kreps *et al.*, 2000) and a large increase in transcription was observed progressively through the light period in both temperature treatments in all genotypes (data not shown). Sac-5 was unique amongst the genotypes for exhibiting an increase in expression in response to chilling (Fig. 4D). Previous reports on the expression of *RD19a* are mixed with some reporting cold induction (Robinson and Parkin, 2008) and some reporting only drought induction (Koizumi *et al.*, 1993; Coupe *et al.*, 2003). In Sac-5, transcription reached a 2.7-fold increase at 12 °C compared with 28 °C 4 h after transfer and then declined.

LER

All genotypes showed a decline in LER after 4 d at 12 °C, although in Gig-311 this difference was not significant (Fig. 6). Sac-5 and Goliath showed the greatest reduction in LER at 12 °C with reductions of 97 % and 86 %, respectively. In both EMI-11 and Gig-311 a slight increase in LER was observed after treatment for 24 h at 12 °C compared with the control plants maintained at 28 °C. In Goliath there was a 79 % reduction in plants treated to 24 h at 12 °C compared with warm-grown plants, indicating that short-term chilling had a lasting detrimental effect on LER.

DISCUSSION

Photosynthesis and carbohydrate phenotypes under optimal growing temperatures

Under optimal growing conditions the rate of photosynthesis (A_{sat}) was highest for Sac-5 and Gig-311 throughout the experiment. The two *M. sinensis* genotypes, particularly EMI-11 were, comparably, low. Previous reports have described the superiority of the photosynthesis rates at lower temperatures in *M. × giganteus* compared with maize (Naidu *et al.*, 2003; Wang *et al.*, 2008a; Dohleman and Long, 2009). In field trials in Illinois, *M. × giganteus* owes its high productivity to a high

green leaf area index and the ability to develop a functional canopy at lower temperatures (Dohleman and Long, 2009). The four genotypes used in this study were selected based, in part, upon their differing morphology. Therefore, despite the lower rate of photosynthesis in individual leaves of the *M. sinensis* genotypes, the higher stem density could still result in a greater capacity for carbon fixation on a per plant basis. Studies are currently underway to investigate this hypothesis.

Under warm conditions, soluble carbohydrates and starch showed a diurnal pattern of accumulation similar to that reported in other species such as maize, cotton and soybean (Huber *et al.*, 1984; Hendrix and Huber, 1986; Kalt-Torres *et al.*, 1987). Sac-5 and EMI-11 showed the most contrasting carbohydrate phenotypes. *Miscanthus sacchariflorus* genotypes are phylogenetically more closely related to sugarcane than *M. sinensis* (Hodkinson *et al.*, 2002) and many aspects of the difference in the carbohydrate phenotype of Sac-5 and EMI-11 reflect this. For example, the reported mid-day concentration of sucrose in sugarcane leaves is 48 mg g⁻¹ d. wt compared with 40 mg g⁻¹ d. wt in Sac-5 (Iskandar *et al.*, 2011). In EMI-11, mid-day sucrose levels were 7 mg g⁻¹ d. wt which is more similar to sucrose concentrations reported in maize of 8 mg g⁻¹ d. wt (Kalt-Torres *et al.*, 1987). In terms of sucrose content, Sac-5 is therefore more similar to sugarcane and EMI-11 is more similar to maize. In Sac-5 the pattern of soluble sugar accumulation is also comparable to sugarcane (Lehrer *et al.*, 2007), with concentration peaking around mid-day and then declining. The pattern of accumulation in EMI-11 showed a progressive increase in soluble sugars from mid-day as has also been reported in the temperate grasses barley and switchgrass (Greenfield and Smith, 1974; Sicher *et al.*, 1984).

Physiological responses of Miscanthus to chilling

After transfer to 12 °C, all of the genotypes suffered a large and rapid decline in photosynthesis. At the same time, an increase in soluble sugar concentration was observed in all genotypes although, proportionately, less so in Sac-5 and Gig-311. The accumulation of soluble sugars and starch is probably the result of the combination of the remaining photosynthesis and a decline in growth rate, reducing carbon utilization. In

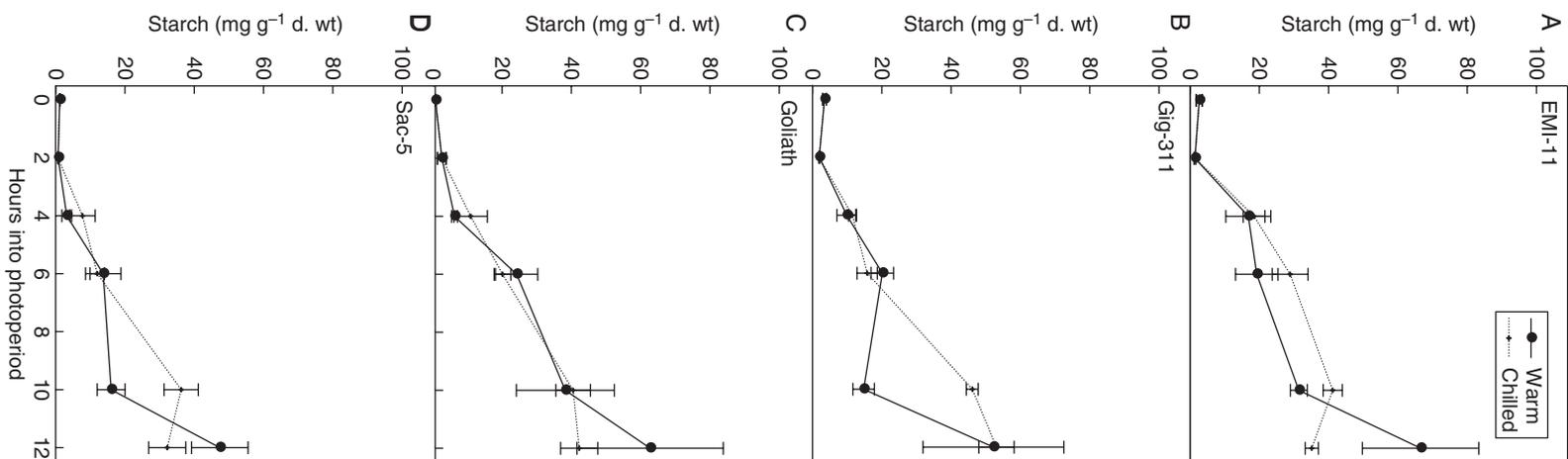


FIG. 3. Starch contents at 28 °C (Warm) and at 12 °C (Chilled) in the four genotypes. Values are means \pm s.e., $n = 3$.

TABLE 4. Transcripts selected for expression analysis

Gene name	Annotation	GI	Reference	<i>Miscanthus</i> gene name
<i>PGM1</i> : <i>Arabidopsis thaliana</i>	<i>PHOSPHOGLUCOMUTASE1</i> (chloroplastic)	15242191	Caspar <i>et al.</i> (1985)	<i>MsPGM1</i>
<i>PGM1</i> : <i>Zea mays</i>	<i>PGM1</i> ; phosphoglucomutase 1 (cytoplasmic)	162463106	Manjunath <i>et al.</i> (1998)	
<i>Sb_03g028080</i> : <i>Sorghum bicolor</i>	<i>PGM1</i> ; phosphoglucomutase 1	242058041		
<i>BAM3/BMY9</i> : <i>Arabidopsis thaliana</i>	<i>BAM3/BMY9</i> chloroplastic β -amylase	18414813	Lao <i>et al.</i> (1999)	<i>MsBAM3</i>
<i>Beta-amylase</i> : <i>Zea mays</i>	β -Amylase 'glycosyl hydrolase family 14 protein'	195615574		
<i>Sb_01g028700</i> : <i>Sorghum bicolor</i>	'Similar to Glycosyl hydrolase family 14 protein'	242035041		
<i>CBF3/DREB1a</i> : <i>Arabidopsis thaliana</i>	<i>DEHYDRATION-RESPONSIVE ELEMENT-BINDING1a</i>	18416559	Yamaguchi-Shinozaki and Shinozaki (1994)	<i>MsDREB1a</i>
<i>DREB1D</i> : <i>Zea mays</i>	<i>DEHYDRATION RESPONSIVE ELEMENT BINDING1D</i>	226507144	Hao <i>et al.</i> (2010)	
<i>Sb_06g025900</i> : <i>Sorghum bicolor</i>	'Hypothetical protein. DNA binding domain found in transcriptional regulators in plants'	242074048		
<i>RD19A</i> : <i>Arabidopsis thaliana</i>	Cysteine proteinase <i>RESPONSIVE TO DEHYDRATION19A</i>	18420375	Koizumi <i>et al.</i> (1993)	<i>MsRD19a</i>
<i>CCP1</i> : <i>Zea mays</i>	<i>CYSTEINE PROTEASE1</i>	162459555	Chevalier <i>et al.</i> (1995); Domoto <i>et al.</i> (1995)	
<i>Sb_04g017830</i> : <i>Sorghum bicolor</i>	'Hypothetical protein. Similar to cysteine proteinase 1 precursor'	242061538		

Genes involved in carbohydrate metabolism and/or cold-stress response in other species were selected from published literature. Primer sequences are listed in Supplementary Data Table S2.

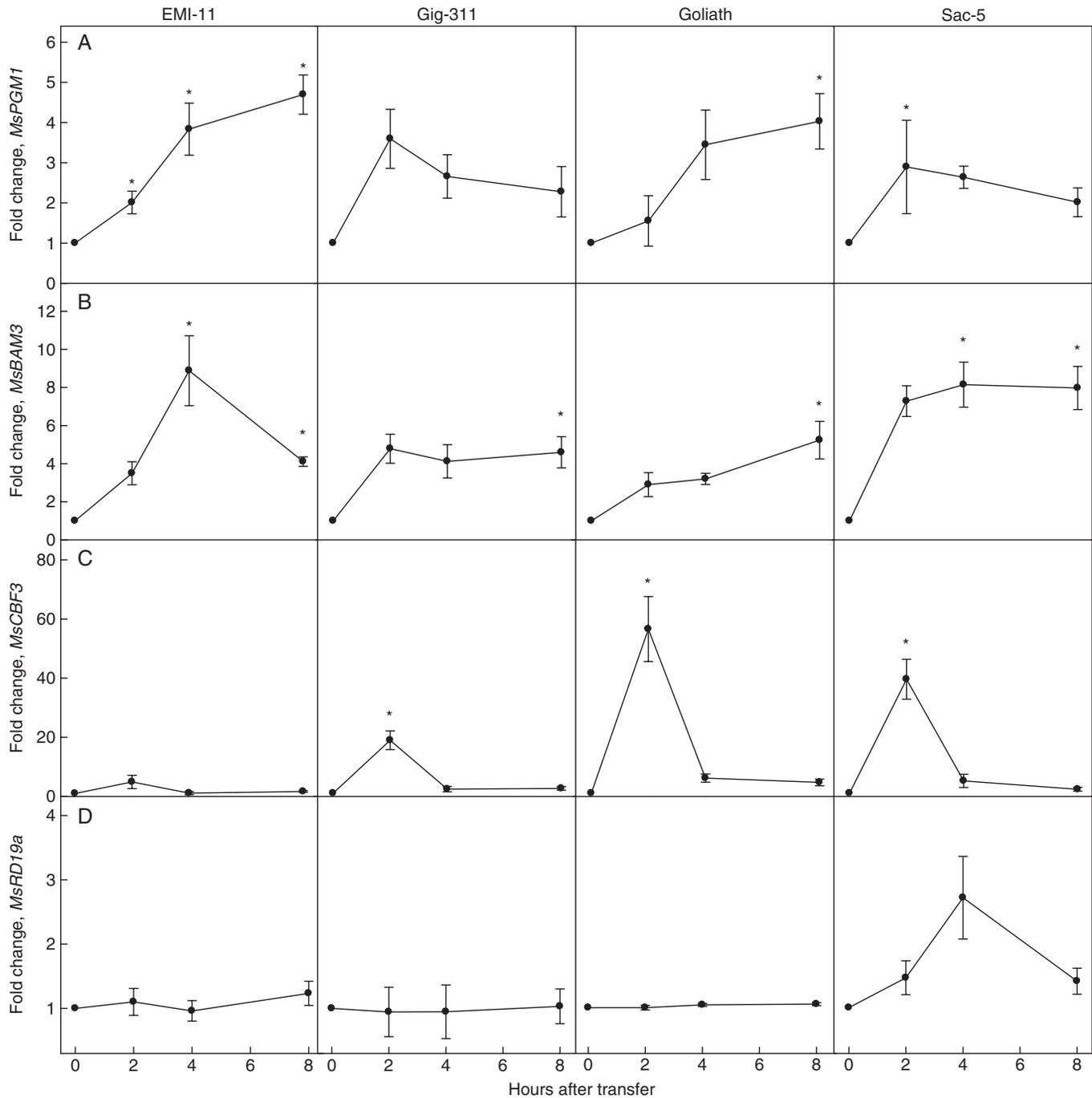


FIG. 4. Fold change in expression between cold/warm conditions of (A) *MsPGM1*, (B) *MsBAM3*, (C) *MsCBF3* and (D) *MsRD19a*. Expression levels at 28 °C and 12 °C were calculated relative to an internal control gene (*MsYLS8*). Fold change is the relative expression at 12 °C/relative expression at 28 °C. Values are means \pm s.e., $n = 3$. * Significant differences to 28 °C (Tukey test, $P < 0.05$). Primer sequences are listed in Supplementary Data Table S2.

sunflower plants grown at 30 °C and then abruptly shifted to 13 °C a similar increase in soluble sugars and starch was observed as in our study (Paul *et al.*, 1991). Carbon labelling demonstrated that carbohydrate export was greatly reduced following the application of chilling stress, and the authors concluded that in chilling conditions assimilation exceeds utilization resulting in carbohydrate accumulation (Paul *et al.*, 1991). A study by Clifton-Brown and Jones (1997) showed that at progressively

cooler temperatures the leaf elongation rate of many genotypes including Gig-311 and Sac-5 were reduced. The reduction in demand for sugars for growth during chilling therefore explains the observations. The two genotypes that suffered the greatest percentage decline in photosynthesis were Goliath and Sac-5. These two genotypes also showed both a large decline in LER after 4 d at 12 °C and reduced LER after being returned to 28 °C after only 24 h in chilling temperatures. Therefore either

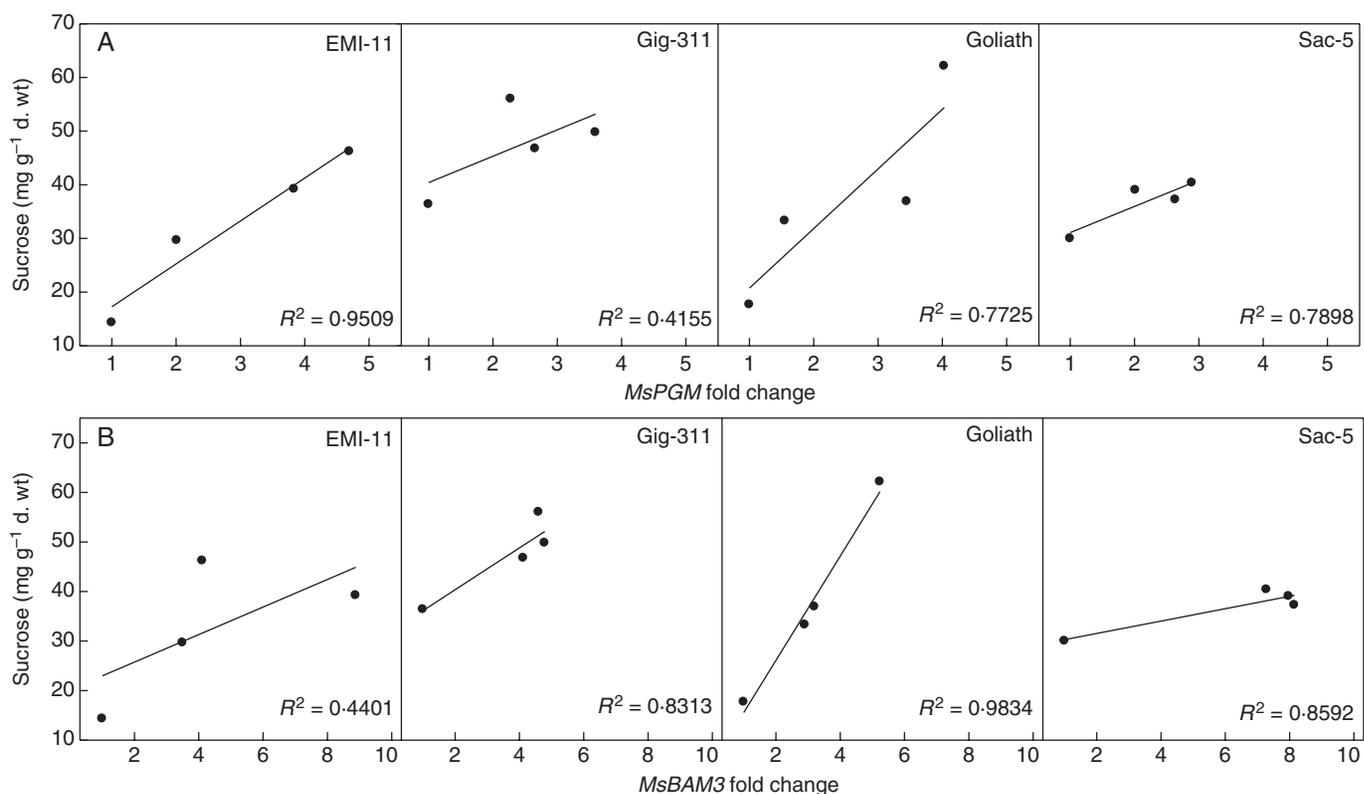


FIG. 5. Pearson's correlation coefficient for fold changes in (A) *MsPGM1* and (B) *MsBAM3* against sucrose content after transfer to 12 °C; $n = 3$. Time-points correspond to T0, 2, 4 and 8 h after transfer to 12 °C. Fold change in relative gene expression is as described in Fig. 4.

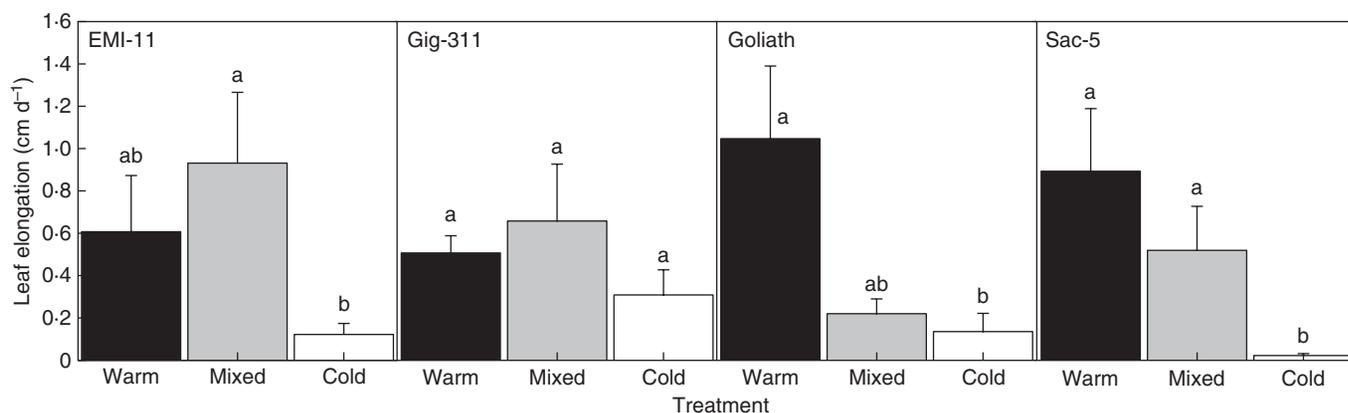


FIG. 6. Average leaf elongation rate of the four genotypes at 28 °C for 4 d (Warm), 12 °C chilling-shock for 24 h before a return to 28 °C for 3 d (Mixed), 12 °C chilling-shock maintained for 4 d (Cold). Values are means + s.e., $n = 4-6$. Different letters show significant differences between treatments for the same genotype (Student's two-tailed t -test, $P < 0.05$).

percentage decline in A_{sat} in the first 4–8 h after chilling or a combination of low LER during chilling and a failure to recover LER were suitable measurements of chilling-shock sensitivity in *Miscanthus*.

The failure of Sac-5 to accumulate sucrose during chilling-shock is similar to the observations of Du and Nose (2002) who reported that a chilling sensitive variety of sugarcane failed to accumulate soluble sugars (including hexoses) in response to chilling. That Sac-5 accumulated large amounts

of hexose in response to chilling but not sucrose indicates that the function of sucrose phosphate synthase (SPS) and/or cFru-1,6-BPase (FBPase) may have been impaired by chilling. In the chilling-sensitive sugarcane genotype a decrease in SPS enzyme activity was observed after only 4 h of exposure to chilling, whereas no difference was observed in tolerant genotypes (Du and Nose, 2002). Furthermore, in maize plants exposed to short-term chilling conditions, FBPase activity was reduced by half even after a return to optimal conditions,

demonstrating lasting damage to the enzyme (Kingston-Smith *et al.*, 1997). The superior photosynthetic performance of *M. × giganteus* compared with maize under chilling conditions has been attributed, in part, to the increased abundance of pyruvate orthophosphate dikinase (PPDK) (Naidu *et al.*, 2003; Wang *et al.*, 2008b). The poorer photosynthetic performance during chilling of EMI-11, Goliath and Sac-5, compared with Gig-311 may reflect a reduced ability to retain activity or abundance of PPDK during chilling. However, our results also suggest that the resilience of other enzymes, such as SPS or FBPase, may also be indicative of the performance of different genotypes of *Miscanthus* under chilling conditions.

In arabidopsis the accumulation of soluble sugars has been attributed, at least in part, to the hydrolysis of starch (Kaplan and Guy, 2004, 2005; Yano *et al.*, 2005). In *Miscanthus* none of the increase in soluble sugars after only a 2-h exposure to chilling conditions could be attributed to starch breakdown as starch continued to accumulate until at least 8 h after exposure. The rate of starch accumulation following chilling was actually greater than at 28 °C between 6 and 8 h in all genotypes except Goliath. This response has also been reported in maize (Hodges *et al.*, 1997) and sunflower (Paul *et al.*, 1991) but the contrary has been observed in arabidopsis (Yano *et al.*, 2005; Kaplan and Guy, 2004, 2005). Both maize and sunflower are sensitive to chilling and freezing, although genotypic variation in tolerance exists for both (Hodges *et al.*, 1997; Allen and Ort, 2001; Balbuena *et al.*, 2011). However, arabidopsis is tolerant to freezing as long as a period of acclimation is first experienced (Thomashow, 1999). The reported Lt_{50} (the temperature at which the calculated percentage damage would exceed 50 %) for acclimated arabidopsis is -7 to -12 °C (Hannah *et al.*, 2006) which is colder than the Lt_{50} for shoots of acclimated *Miscanthus* genotypes which ranges between -6 °C and -9 °C (Farrell *et al.*, 2006). In arabidopsis the breakdown of starch following exposure to chilling conditions has been correlated with freezing tolerance (Kaplan and Guy, 2005). In *Miscanthus*, maize and sensitive genotypes of sunflower, the absence of this response may be representative of their greater freezing susceptibility.

Consistent with previous reports of the greater tolerance of *M. × giganteus* to sub-optimal temperatures (Naidu *et al.*, 2003; Farage *et al.*, 2006; Wang *et al.*, 2008a), Gig-311 maintained the highest photosynthesis rates and showed no reduction in LER in response to chilling. A number of research groups, from different countries, have observed superiority in the performance of *M. × giganteus* genotypes for many different traits. This is particularly interesting as they do not all represent the same clone. A recent study into the performance of different genotypes of *Miscanthus* in Poland included two triploid *M. × giganteus* originating from separate hybridization events (Jezowski *et al.*, 2011). Both *M. × giganteus* genotypes yielded more than the *M. sinensis* intraspecific hybrids and the interspecific *M. sacchariflorus* × *M. sinensis* diploid hybrids (Jezowski *et al.*, 2011). This finding, along with our own and the numerous other reports of the superior performance of *M. × giganteus* (Clifton-Brown and Jones, 1997; Naidu *et al.*, 2003; Naidu and Long, 2004; Wang *et al.*, 2008a, b; Dohleman and Long, 2009; Jezowski *et al.*, 2011), provides evidence for the release of hybrid vigour in the triploid progeny of tetraploid *M. sacchariflorus* × diploid *M. sinensis*.

Gene expression in response to chilling

In arabidopsis the expression of β -amylase is known to be stimulated by cold (Nielsen *et al.*, 1997; Kaplan and Guy, 2004) and a high sugar status (Mita *et al.*, 1995, 1997). Whether induction during cold stress is actually the result of sugar accumulation rather than induction being directed through a cold-signalling pathway, is unclear. We observed a strong correlation between the increase in expression of the two carbohydrate-metabolizing transcripts, *MsPGMI* and *MsBAM3*, and the accumulation of sucrose in each genotype, suggesting that this was the cause of induction. However, the greatest fold-change in expression of *MsBAM3* was observed in Sac-5, which had the lowest increase in soluble sugar of the four genotypes, suggesting that the sugar responsiveness of *MsBAM3* varies between genotypes. Different degrees of increased expression for a β -amylase gene have also been observed in arabidopsis with the Landsberg *erecta* accession showing reduced expression compared with Columbia after sugar feeding (Mita *et al.*, 1997). This indicates that allelic variation controls induction of β -amylase in response to increasing sugar contents which may account for the difference observed here. It is interesting to note that the highest increase in expression of *MsBAM3* was observed in Sac-5 and EMI-11, and these were the only genotypes to show a reduced quantity of starch at the end of the experiment. The expression level of neither *MsBAM3* nor *MsPGMI* strongly reflected the degree of chilling-shock sensitivity in the four genotypes tested; therefore we conclude that these transcripts are not suitable markers of sensitivity.

Arabidopsis *RD19a* has been reported to be cold inducible (Robinson and Parkin, 2008) but more often as being specifically drought responsive (Koizumi *et al.*, 1993; Coupe *et al.*, 2003). Sac-5 was the only genotype to show an increase in expression of this transcript in response to chilling. Although the plants used in this study were kept well watered throughout the experiment, during chilling, water stress may occur in chilling-sensitive plants due to reduced xylem hydraulics which can drastically impair water uptake (Allen and Ort, 2001). This is particularly the case if the roots cool more quickly than the above-ground tissues (Allen and Ort, 2001). To this effect, rhizome shape could affect chilling tolerance; *M. sacchariflorus* genotypes (including Sac-5) have a spreading-rhizome morphology, whereas *M. sinensis* and Gig-311 have compact 'tussock' rhizomes (Clifton-Brown and Lewandowski, 2000). A spreading morphology may expose a greater surface area for rapid cooling than the compact form, resulting in acute water stress and the subsequent expression of drought-responsive genes during chilling-shock.

The CBF/DREB family of transcription factors are rapidly induced in response to cold and activate the expression of a large family of cold-inducible genes (Gilmour *et al.*, 1998; Fowler and Thomashow, 2002). In arabidopsis sequencing of *CBF1*, -2 and -3 from accessions from a latitudinal gradient revealed polymorphisms, particularly amongst southern European-derived accessions compared with northern-origin accessions. The observed polymorphisms affected the expression of downstream genes with southern accessions showing the lowest level of expression and the greatest susceptibility to freezing (Zhen and Ungerer, 2008). Our study identified that *MsCBF3* was most highly expressed in the genotypes that showed the greatest percentage

reduction in A_{sat} and LER and recovery. Studies are now underway to determine whether expression level and/or sequence polymorphisms of *MsCBF3* are a good indicator of chilling tolerance or susceptibility in a larger selection of *Miscanthus* plants originating from a latitudinal gradient in Asia. If, similarly to arabidopsis, gene polymorphisms relating to tolerance are identified, this gene may be used as a molecular marker to screen seedlings and potential parents for chilling tolerance.

In summary: under optimal conditions the *M. sacchariflorus* genotype showed a diurnal pattern of carbohydrate accumulation that was similar to sugarcane, whereas the *M. sinensis* genotypes more closely resembled temperate grasses. In keeping with its reported superior performance in prolonged cool temperatures, *M. × giganteus* also maintains a greater degree of resilience to chilling-shock than other genotypes. The failure of *M. sacchariflorus* (Sac-5) to accumulate sucrose indicates impairment in the enzyme function of SPS or FBPase during chilling. The most chilling-sensitive genotypes most highly expressed the candidate transcription factor, *MsCBF3*, suggesting that this gene may be a suitable molecular marker of chilling tolerance or susceptibility.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: temperature changes during daylight hours in May 2010 at Aberystwyth, UK. Table S2: list of primer sequences used in the study.

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